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Abstract: Background: Effects of elevated atmospheric CO₂ concentrations on plant growth and associated C cycling have intensively been studied, but less is known about effects on the fluxes of radiatively active trace gases other than CO₂. Net soil-atmosphere CH₄ fluxes are determined by the balance of soil microbially-driven methane (CH₄) oxidation and methanogenesis, and both might change under elevated CO₂. Methods and Results: Here, we studied CH₄ dynamics in a permanent grassland exposed to elevated CO₂ for 14 years. Soil-atmosphere fluxes of CH₄ were measured using large static chambers, over a period of four years. The ecosystem was a net sink for atmospheric CH₄ for most of the time except summer to fall when net CH₄ emissions occurred. We did not detect any elevated CO₂ effects on CH₄ fluxes, but emissions were difficult to quantify due to their discontinuous nature, most likely because of ebullition from the saturated zone. Potential methanotrophic activity, determined by incubation of fresh sieved soil under standardized conditions, also did not reveal any effect of the CO₂ treatment. Finally, we determined the spatial micro-distribution of methanotrophic activity at less than 5× atmospheric (10 ppm) and elevated (10000 ppm) CH₄ concentrations, using a novel auto-radiographic technique. These analyses indicated that domains of net CH₄ assimilation were distributed throughout the analyzed top 15 cm of soils, with no dependence on CH₄ concentration or CO₂ treatment. Conclusions: Our investigations suggest that elevated CO₂ exerts no or only minor effects on CH₄ fluxes in the type of ecosystem we studied, at least as long as soil moisture differences are small or absent as was the case here. The autoradiographic analyses further indicate that the spatial niche of CH₄ oxidation does not shift in response to CO₂ enrichment or CH₄ concentration, and that the same type of methanotrophs may oxidize CH₄ from atmospheric and soil-internal sources.

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RESEARCH ARTICLE

Effects of Long-Term CO₂ Enrichment on Soil-Atmosphere CH₄ Fluxes and the Spatial Micro-Distribution of Methanotrophic Bacteria

Saeed Karbin¹, Cécile Guillet², Claudia I. Kammann^{2,3*}, Pascal A. Niklaus^{1*}

1 Institute of Evolutionary Biology and Environmental Studies, University of Zürich, Zürich, Switzerland, **2** Institute of Plant Ecology, Justus-Liebig-University, Giessen, Germany, **3** Climate Change Research for Special Crops, Hochschule Geisenheim University, Geisenheim, Germany

* Pascal.Niklaus@ieu.uzh.ch(PN); Claudia.Kamman@hs-gm.de(CK)



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Abstract

Background

Effects of elevated atmospheric CO₂ concentrations on plant growth and associated C cycling have intensively been studied, but less is known about effects on the fluxes of radiatively active trace gases other than CO₂. Net soil-atmosphere CH₄ fluxes are determined by the balance of soil microbially-driven methane (CH₄) oxidation and methanogenesis, and both might change under elevated CO₂.

Methods and Results

Here, we studied CH₄ dynamics in a permanent grassland exposed to elevated CO₂ for 14 years. Soil-atmosphere fluxes of CH₄ were measured using large static chambers, over a period of four years. The ecosystem was a net sink for atmospheric CH₄ for most of the time except summer to fall when net CH₄ emissions occurred. We did not detect any elevated CO₂ effects on CH₄ fluxes, but emissions were difficult to quantify due to their discontinuous nature, most likely because of ebullition from the saturated zone. Potential methanotrophic activity, determined by incubation of fresh sieved soil under standardized conditions, also did not reveal any effect of the CO₂ treatment. Finally, we determined the spatial micro-distribution of methanotrophic activity at less than 5× atmospheric (10 ppm) and elevated (10000 ppm) CH₄ concentrations, using a novel auto-radiographic technique. These analyses indicated that domains of net CH₄ assimilation were distributed throughout the analyzed top 15 cm of soils, with no dependence on CH₄ concentration or CO₂ treatment.

Conclusions

Our investigations suggest that elevated CO₂ exerts no or only minor effects on CH₄ fluxes in the type of ecosystem we studied, at least as long as soil moisture differences are small or absent as was the case here. The autoradiographic analyses further indicate that the

spatial niche of CH₄ oxidation does not shift in response to CO₂ enrichment or CH₄ concentration, and that the same type of methanotrophs may oxidize CH₄ from atmospheric and soil-internal sources.

Introduction

The atmospheric concentrations of greenhouse gases including carbon dioxide (CO₂) and methane (CH₄) have increased since pre-industrial times due to anthropogenic activities. A question of particular concern is how elevated atmospheric CO₂ concentrations affect terrestrial ecosystems and their functioning. Studies of plant growth responses and of effects on the carbon balance of ecosystems have dominated elevated CO₂ research to date. However, although CO₂-effects are solely mediated by the plant's photosynthetic apparatus, elevated CO₂ can influence virtually every plant or microbial process through alterations of the ecosystem's carbon, nitrogen or water dynamics. An intriguing question is whether these effects will affect the ecosystem's balance of trace gases other than CO₂ such as CH₄. Such a mechanism would interact with global climatic change, similar to effects on carbon sequestration.

The CH₄ balance of an ecosystem is determined by the sum of sources and sinks, both of which are almost exclusively driven by soil microbial processes [1] (but see [2, 3]). Whether sources or sinks dominate is often determined by oxygen availability, with CH₄ oxidizing micro-organisms driving soil CH₄ uptake under aerobic conditions whereas methanogenesis by archaea dominates under anaerobic conditions, e.g. in waterlogged soils. Methanogenesis and CH₄ oxidation often co-occur, with a substantial fraction of the CH₄ produced in anoxic soil domains being consumed by methanotrophs before it diffuses to the atmosphere. Under these conditions, methanotrophs functionally act as a "biofilter" for endogenous CH₄. Conversely, methanogenesis can prime the activity of methanotrophs [4], which then in turn will oxidize larger amounts of atmospheric CH₄ once the soil-internal sources cease [5]. Oxidation of atmospheric CH₄ (low concentrations) or soil-internal CH₄ (high concentrations) requires enzymes with vastly different kinetic properties. Methanotrophic organisms growing at atmospheric CH₄ concentrations have not been isolated to date, and it therefore remains unclear whether different groups of methanotrophs are responsible for these two sinks or whether the same organisms exhibit different CH₄ oxidation kinetics by physiological adjustment [5].

The ecology of atmospheric CH₄ oxidation is not well understood to date. Many studies have shown that gas phase diffusive CH₄ transport limitations often control soil CH₄ uptake, at least at moderate to high soil moisture [6]. However, moisture can also limit methanotrophic activity due to physiological stress [7]. A second important factor is nitrogen availability. High mineral nitrogen levels, in particular NH₄⁺, can inhibit CH₄ oxidation. Laboratory studies have attributed this effect to inhibition of methane mono-oxygenase, the enzyme catalyzing the first step of CH₄ assimilation. However, mineral N also is an essential nutrient and the relationship between CH₄ oxidation and N levels therefore is more complicated [8]. Finally, inhibition of methanotrophic activity does not necessarily translate into reduced soil CH₄ uptake. [9] have demonstrated that mineral fertilizer N that accumulates under drought (because plant uptake is reduced) can inhibit methanotrophs in the top soil layers, but that methanotrophs in deeper soil layers can compensate for this loss of function (because diffusion is facilitated by low soil moisture), so that no effect manifests in soil surface CH₄ fluxes.

Elevated CO₂ concentrations have the potential to affect soil CH₄ transformations by various mechanisms. First, CO₂-enrichment is often found to increase soil moisture due to

increased photosynthetic water use efficiency [10, 11]. Since soil moisture is an important controller of CH₄ diffusion rates, CH₄ oxidation could be reduced by this mechanism. Second, elevated CO₂ can reduce mineral N availability through increased plant and microbial N uptake and through effects on microbial N transformation rates [12–15], which in turn might alter CH₄ oxidation. Third, plants exposed to elevated CO₂ can produce larger amounts of organic compounds that enter the soil via rhizodeposition and litterfall [16]. These could fuel methanogenesis through higher substrate availability and lower redox potential caused by higher respiration rates. Some of these compounds could also directly inhibit methanotrophs, since inhibitory effects have been demonstrated for ethylene [17], some organic acids [18], and terpenes [19].

We studied soil-atmosphere CH₄ fluxes in a grassland that had been exposed to elevated CO₂ using free-air CO₂ enrichment (FACE) for 14 years [20]. Fluxes were assessed with large static chambers. We further determined the spatial micro-distribution of methanotrophs that actively assimilated CH₄ under low and high CH₄ concentrations, using a novel auto-radio-graphic technique. These investigations addressed the following questions: (1) does elevated CO₂ affect soil-atmosphere CH₄ fluxes? (2) Does the spatial micro-distribution of active methanotrophs change under elevated CO₂, and can such effects be related to the observed system-level fluxes? (3) Is the spatial niche of active methanotrophs oxidizing CH₄ originating from the atmosphere or from soil-internal sources different?

Methods

Study site and experimental design

We studied effects of long-term elevated atmospheric CO₂ on soil-atmosphere CH₄ fluxes and the micro-distribution of methanotrophic bacteria in a permanent grassland near Giessen, Germany (50°32' N and 8°41.3' E, 172 m a.s.l.). For at least the past 50 years, the site has been permanent grassland fertilized with 50–80 kg N ha⁻¹. From 1995 onwards, fertilization was reduced to 40 kg N ha⁻¹ a⁻¹ (see [20] for further details).

In 1997, three circular plot pairs (FACE rings with 8 m inner diameter) were established. One plot per pair was selected randomly and atmospheric CO₂ enriched to 20% above ambient conditions during daylight hours since May 1998, using free-air CO₂ enrichment (FACE). The other plot of the pair served as ambient CO₂ control.

Vegetation at the site is classified as Arrhenatheretum elatioris Br.-Bl. [21] and contains about 60 vascular plant species [20]. The soil is a Fluvic Gleysol with sandy loam texture over clay. The top soil is slightly acidic (pH of 6.0) and has an organic C content of 4.6% and 3.6% in 0–5 and 5–15 cm depth [20].

In situ soil-atmosphere CH₄ fluxes

From 2009 to 2012, we measured soil-atmosphere CH₄ fluxes on a total of 191 days *in situ* with large static chambers (94 cm inner diameter, ca. 160L volume; modified according to [22]; for further details see [7]). We collected three 25 mL headspace samples at 30 minute intervals and analyzed these by gas chromatography. CH₄ fluxes were estimated by linear regression of concentrations against time. We accepted all measurements with a residual standard error (RSE) of less than 15 ppb CH₄, plus the measurements where the ratio of RSE to calculated flux indicated that omission of any of the three points would have changed the result by less than 20%. Measurements that did not fulfil these criteria were analyzed separately, using other methods, as is discussed in the results section.

Soil moisture and water table depth

Soil moisture was recorded automatically at 4 locations per plot using TDR-probes (P2G, 0–15 cm depth, Imko, Ettlingen, Germany). Water table depth was recorded manually on each weekday, using three custom-built water-level gauges that were placed between pairs of ambient and elevated CO₂ plots.

Soil sampling

On July 6 and October 25, 2011, we harvested two intact soil cores per plot. Cores were sampled with PVC tubes (20 cm depth x 6.5 cm internal diameter) that were driven 15 cm into the soil. In order to minimize soil compaction, the top soil had first been pre-cut along the tube's circumference with a knife. Cores were then capped at both ends to prevent water loss.

On July 6, 2011, we further collected soil at two random locations per plot. These samples were divided by five centimeter depth interval, down to a depth of 20 cm. The two replicate samples per plot were combined per depth layer and transported to the laboratory for further analysis.

CH₄ oxidation of sieved soil samples

We sieved the soil samples (2 mm mesh) and determined soil moisture gravimetrically (5 g fresh soil, 105°C, 24 h). Fresh soil equivalent to 100 g dry weight per plot and depth layer was incubated at 20°C in 1 L air-tight glass jars. The jars were ventilated under ambient conditions, and headspace CH₄ concentration determined 0, 2, and 4 h after closing of the lids. CH₄ uptake rates were calculated by linear regression against sampling time.

Radiolabelling of intact soil cores

The intact soil cores collected at the field site were placed in gas-tight 3 L jars (with the bottom end of the tube still capped). The jars were closed and headspace samples analyzed for CH₄ after 0, 2, 4 and 6 h to determine the core's net CH₄ uptake rates.

The jars were then ventilated and the soil cores labelled with ¹⁴CH₄. Two soil cores per plot and sampling date were labelled at slightly above-ambient CH₄ concentrations (max. 10 ppm). Two additional soil cores from the July 6, 2011 sampling were labelled at high CH₄ concentrations (ca. 10000 ppm). The rationale of this procedure was to test for differences in spatial activity distribution under these contrasting conditions. A total ¹⁴C activity of ca. 100kBq was applied over a period of 6 days. Plastic tubes with 100 mL 1M NaOH were placed in each jar to trap CO₂ (incl. ¹⁴CO₂) produced by microbial respiration. We regularly injected O₂ into the jars to maintain O₂ concentrations around 20%.

Then, the soil cores were freeze-dried and impregnated with epoxy resin (Laromin C 260, BASF, Ludwigshafen, Germany, mixed at a ratio of 2:3 with Araldite DY 026SP hardener, Astorit AG, Einsiedeln, Switzerland) as described in [9]. The resin was left curing at room temperature for 3 days, followed by an overnight incubation at 60°C for final hardening. The soil cores were then cut twice vertically using a diamond saw, creating a section of ca. 8 mm thickness. This section was cut into three equal pieces which were glued onto 5 × 5 cm glass carriers and levelled with a diamond cup mill (Discoplan, Struers GmbH, Birmensdorf, Switzerland).

We exposed phosphor imaging plates (BAS III S, Fuji Photo Film Ltd., Tokyo, Japan) to levelled soil sections for 3 days. The imaging plates were then digitized by red-excited fluorescence scanning at a resolution of 200 μm (BAS-1000, Fujix corp., Tokyo, Japan). We corrected the scans for background exposure and recombined the three image sections to a single image of the cross-sectional area of the original soil cores, using custom Matlab scripts (Image

processing toolbox, Matlab, Mathworks, Natick, MA). The sections were inspected visually, and the vertical distribution of the label calculated by averaging pixel values by horizontal pixel line (excluding large stones).

Statistical analysis

The unit of replication for the elevated CO₂ treatment is the field plot. We therefore analyzed the data using one-way ANOVA with CO₂ treatment as fixed effect and field plot ($n = 6$) as replicate. We considered pairs of plots ("block" factor) and the geographical northing and easting to account for spatial variation, but these terms consumed excessive degrees of freedom given the small sample size, and did not change the results, so that we did not include them in the final model. Effects with $P \leq 0.05$ are referred to as significant, effects with $P \leq 0.1$ as marginally significant.

Results

In situ soil-atmosphere CH₄ fluxes

Our static chamber measurements ([S1 Dataset](#)) revealed three characteristic patterns in which CH₄ concentrations evolved over the three headspace samplings ([Fig 1](#)). During the major part of the measurements, concentrations progressed linearly with time ([Fig 1A](#)), either decreasing from ambient to sub-ambient CH₄ concentrations (net soil CH₄ uptake), or increasing to a few hundred to thousand ppb above ambient concentrations (net soil CH₄ emission). However, in other cases, episodic emissions resulted in a sudden increase of concentrations between some of the headspace samplings ([Fig 1B](#), here shown for emission between 1st and 2nd headspace sampling). We refer to these cases as "bubble emission" since they are likely caused by ebullition from deeper soil layers or the water table. Finally, we also observed CH₄ concentrations that were markedly above ambient at the first sampling and decreased thereafter ([Fig 1C](#)). We termed this pattern "redistribution" since it is likely caused by a localized "bubble emission" prior to the first sampling, followed by redistribution of CH₄ in the chamber and soil pore volume. There were also cases suggesting a combination of "bubble emission" and "redistribution", but these were more difficult to classify.

Meaningful emission rates can only be calculated for the linear case ([Fig 1A](#)). In the absence of non-linear emissions, soils were net sinks for CH₄ ([Fig 2A](#), white background). Soil CH₄ uptake during these periods did not differ significantly between CO₂ treatments (26.2 ± 4.7 and 28.6 ± 5.2 $\mu\text{mol CH}_4 \text{ m}^{-2} \text{ d}^{-1}$ in ambient and elevated CO₂, respectively). During periods in which "bubble emissions" occurred ([Fig 2A](#), grey background), average rates determined from the remaining chambers showing linear emissions were generally positive, i.e. indicated net soil CH₄ emissions. These emissions likely are lower bounds of the real fluxes because they do not include the supposedly higher emission rates when "bubbles" are formed.

Soil CH₄ fluxes (excl. periods with "bubble" emissions) were correlated to soil moisture and water table depth, which explained 37% and 57% of the temporal variation in soil-atmosphere CH₄ exchange ($P < 0.001$, two extreme flux values excluded, sampling day as replicate, [Fig 2B and 2C](#)). Soil moisture and water table depth were highly correlated ($r = 0.74$). CH₄ fluxes did not significantly depend on daily precipitation.

Bubble emissions occurred in 14.3 (average of 6 plots) out of 168 samplings, with no significant difference between CO₂ treatments ($P = 0.9$, generalized linear model with binomial distribution). Virtually identical results were obtained when the number of static chambers per plot showing such emissions (0 to 3 per plot) was considered instead of simply discriminating between occurrence and absence on a plot basis.

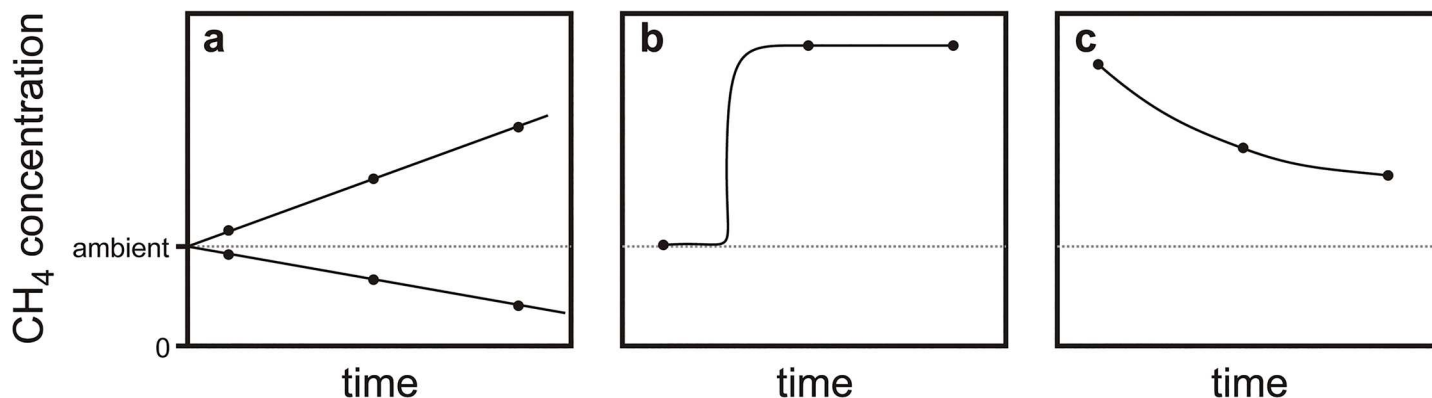


Fig 1. Typical time-courses of CH₄ concentrations during static chamber sampling. (a) Linear concentration changes with time, indicating continuous soil CH₄ uptake or release. (b) Step-increase in CH₄ concentration, likely caused by emission bursts that could originate from ebullition from the underlying saturated zone. (c) Decrease in CH₄ concentrations, starting at substantially above-ambient CH₄ concentrations; this pattern is likely caused by a re-distribution of localized CH₄ emissions trapped in the static chamber.

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CH₄ uptake of incubated soil samples

The sieved 5-cm soil layers did not reveal any effect of CO₂ enrichment when incubated at 20°C and field moisture (Fig 3). Intact soil cores incubated in the laboratory at 20°C also did not show any effect of elevated CO₂ on net CH₄ uptake (Fig 4, volumetric soil moisture content of 23% and 46% on July 6 and October 25, respectively).

¹⁴CH₄ labelling of soil cores

Visual inspection of autoradiographies revealed heterogeneous label assimilation, with distinct zones of enhanced net CH₄ assimilation (Figs 5, 6 and 7). These appeared to be along cracks and around aggregate structures (e.g. Fig 6). On both July 6 and October 25, net ¹⁴CH₄ assimilation was reduced in the top 1–2 centimeters relative to the rest of the soil profile which showed relatively little variation in label intensity with depth.

CO₂ enrichment did not affect the vertical distribution of the label except for an interaction with depth ($P < 0.05$) that originated from lower labelling of the uppermost layer on October 25 when labelled at high CH₄ concentration. Since the analysis of depth x CO₂ treatment includes some degree of autocorrelation of residuals between soil layers, we calculated mean oxidation depth per soil core as

$$\int_y y \cdot a(d) dy / \int_y a(d) dy,$$

i.e. as activity-weighted mean depth of net CH₄ assimilation (Table 1); figuratively, this is the depth of the center of gravity an activity depth profile. Mean assimilation depth averaged 3.8 cm, irrespective of CO₂ treatment and labelling concentration. There was a marginally significant shift of 0.5 cm towards the soil surface in October relative to July 2011 ($P = 0.06$).

Discussion

In the grassland investigated, soil-atmosphere CH₄ fluxes were characterized by alternating phases of soil net CH₄ uptake and emission. On an annual basis, the studied ecosystem was a net source of CH₄, with emissions peaking during the summer months and oxidation prevailing during most of the remaining time. However, the annual CH₄ balance is difficult to constrain due to the “burst” character of emissions which is not amenable to the static chamber

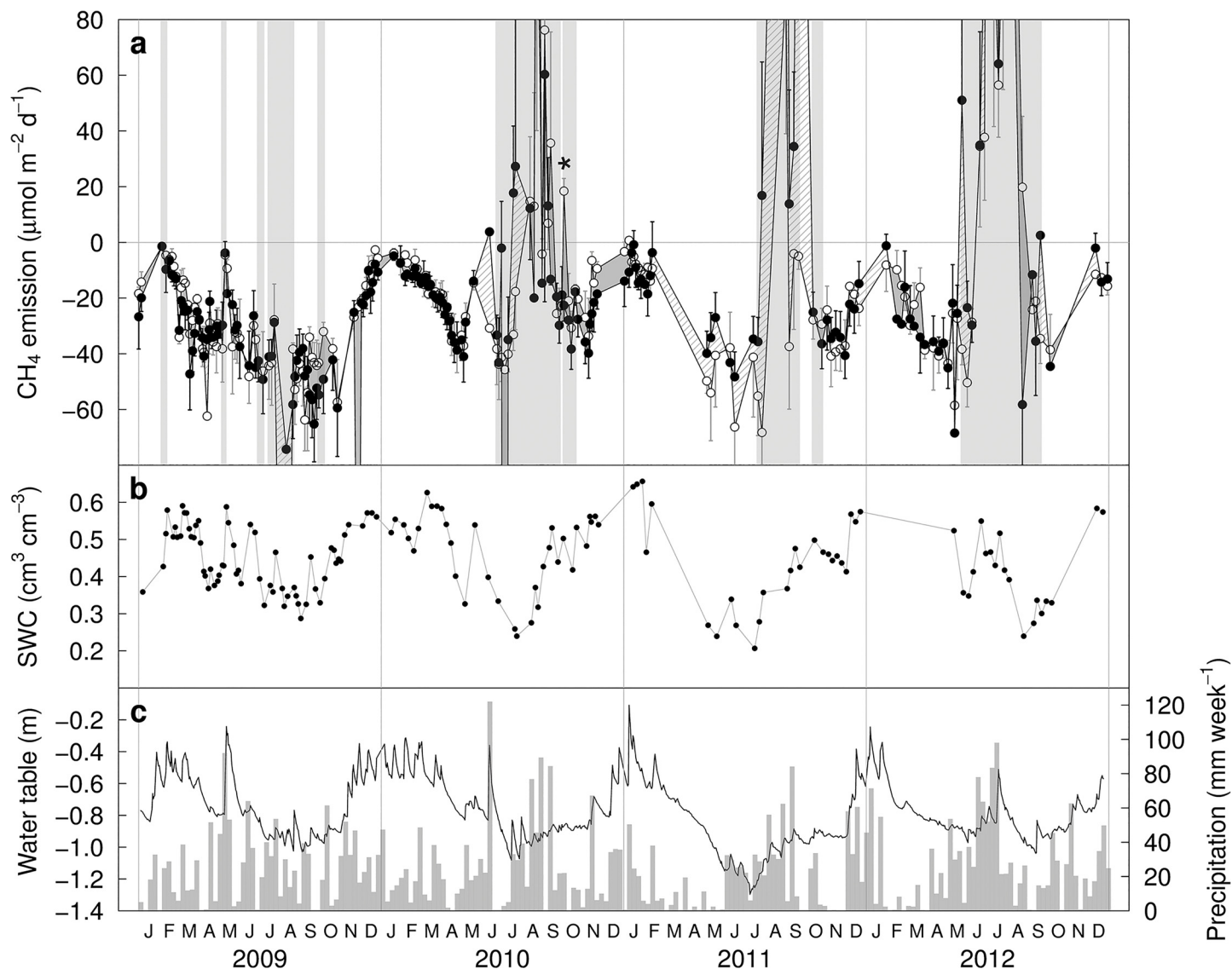


Fig 2. CH₄ fluxes and related environmental data. (a) CH₄ emission rates in ambient (○) and elevated CO₂ (●) plots, calculated when concentration changes were linear (mean ± s.e., $n \leq 3$ per CO₂, depending on the number of plots with emissions following the pattern of Fig 1A). Effects of elevated CO₂ were not statistically significant. Periods during which emissions occurred (Fig 1B and 1C) are shaded in gray, indicating that emission rates likely are underestimates. (b) Volumetric soil moisture, averaged across CO₂ treatments. (c) Weekly precipitation and water table depth.

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technique we adopted. We did not detect any effects of elevated CO₂ on fluxes or micro-distribution of CH₄ assimilation, but this also may be related to the relatively low power originating from the low replication typical of FACE studies.

Evidence regarding effects of elevated CO₂ on CH₄ fluxes is equivocal. In a study in Loblolly pine plantation [23, 24] reductions in soil CH₄ sink were found under CO₂ enrichment, which were related to increased soil moisture due to reduced stomatal conductance and increased water use efficiency [25]. The authors argued that this effect on CH₄ uptake originated from diffusive CH₄ transport limitation in the top soil but possibly also from increased anoxia in deeper soil layers due to higher plant and heterotrophic soil microbial activity, which could promote methanogenesis. Similar effects were found in trembling aspen stands [26]. Interestingly, in semi-arid grassland, opposite effects of elevated CO₂ were found when soils were dry

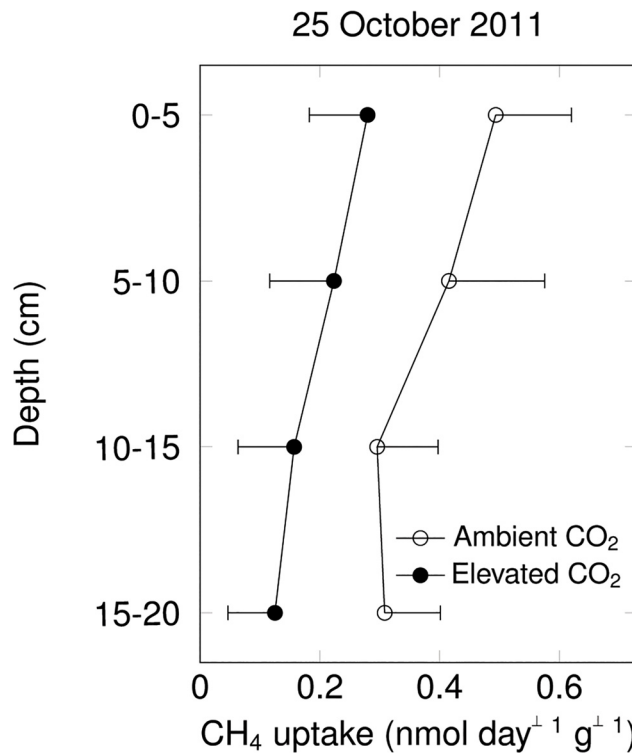


Fig 3. Net CH₄ uptake rates of sieved field-moist soil incubated at 20°C in the laboratory (mean ± s.e., by 5cm soil layer; n = 3 per CO₂ treatment; effects of elevated CO₂ were not statistically significant).

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[27]; the authors attributed these effects to a reduction of drought stress due to moister soils under elevated CO₂. This conclusion was supported by soil CH₄ uptake rates decreasing when soil moisture was above or below some intermediate optimum. However, [28] found reduced CH₄ uptake under elevated CO₂ in a mixed *Lolium*/*Trifolium* sward, and this effect was

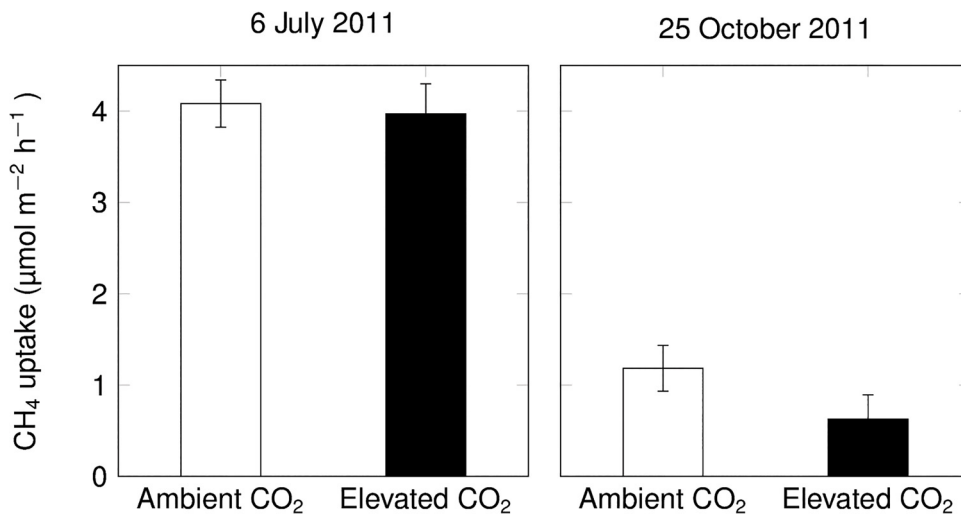


Fig 4. Net CH₄ uptake rates of intact soil cores collected in ambient and elevated CO₂ plots and incubated in the laboratory at 20°C (mean ± s.e., n = 3 per CO₂ treatment; effects of elevated CO₂ were not statistically significant).

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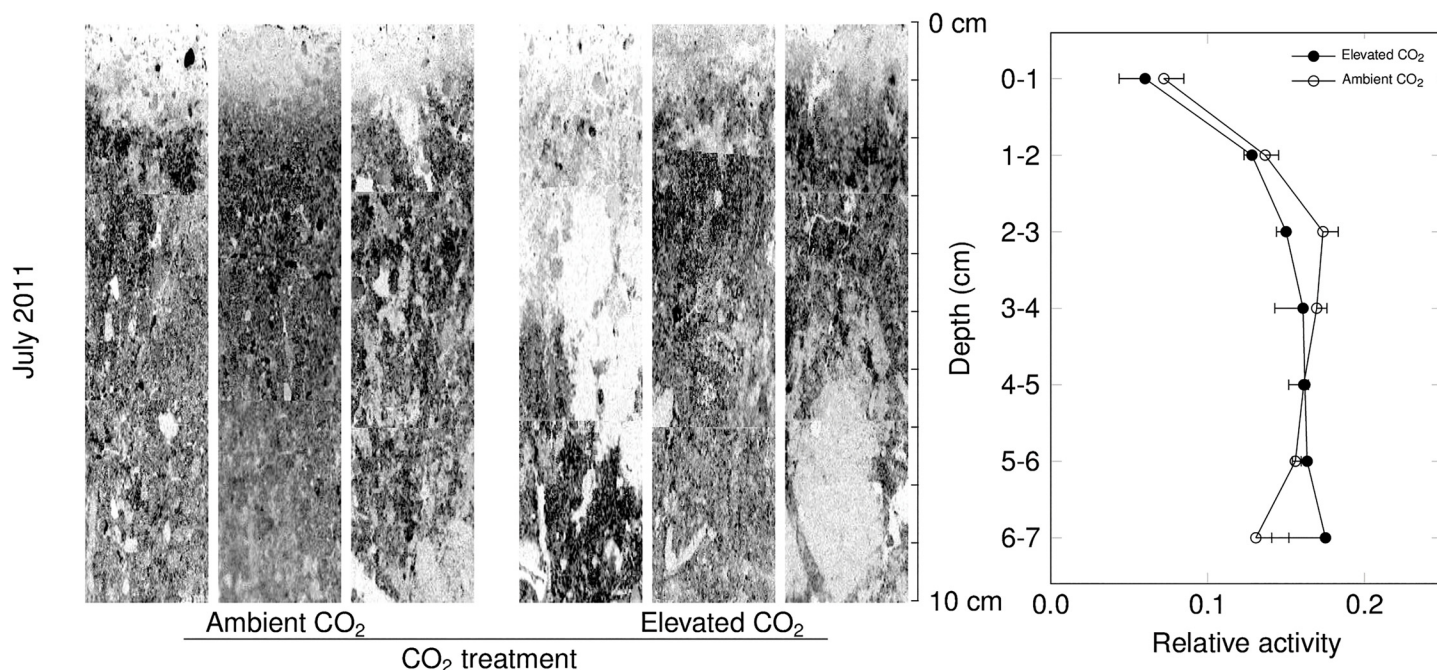


Fig 5. Soil micro-autoradiography of typical soil sections collected on June 6, 2011, and incubated under near-ambient CH₄ concentrations. Darker pixels indicate higher labelling. Vertical profiles of labelling (right panel), aggregated by 1 cm depth intervals (mean \pm s.e., $n = 3$ per CO₂ treatment). Effects of elevated CO₂ were not statistically significant.

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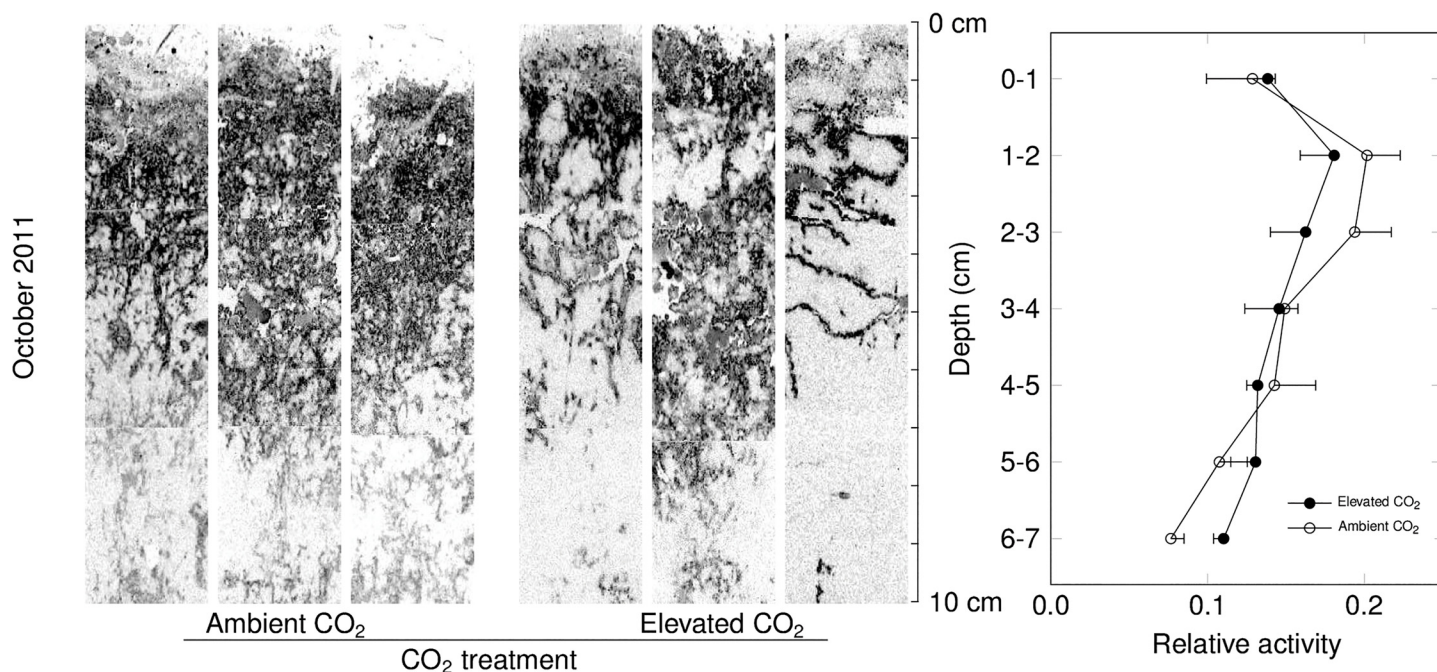


Fig 6. Soil micro-autoradiography of typical soil sections collected on October 25, 2011, and incubated under near-ambient CH₄ concentrations. Darker pixels indicate higher labelling. Vertical profiles of labelling (right panel), aggregated by 1 cm depth intervals (mean \pm s.e., $n = 3$ per CO₂ treatment). Effects of elevated CO₂ were not statistically significant.

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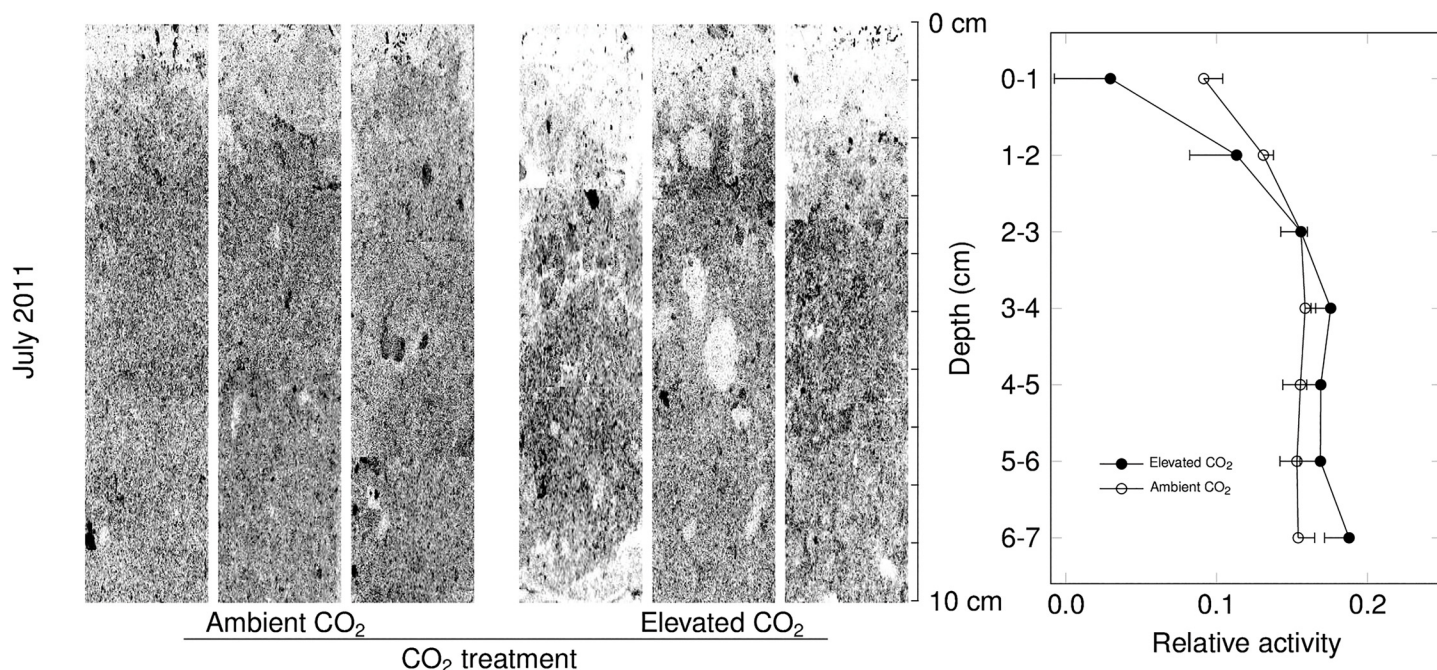


Fig 7. Soil micro-autoradiography of typical soil sections collected on October 25, 2011, and incubated under CH₄ concentrations around 10000 ppm. Darker pixels indicate higher labelling. Vertical profiles of labelling (right panel), aggregated by 1 cm depth intervals (mean \pm s.e., $n = 3$ per CO₂ treatment). Elevated CO₂ marginally significantly affected the depth distribution of methanotrophic activity ($P = 0.06$ for depth \times CO₂).

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unrelated to soil moisture. Finally, CH₄ uptake and CO₂ concentration were unrelated in a number of other studies (wheat: [29], Sorghum and soybean: [30]; shortgrass steppe: [31]). We observed a median net soil CH₄ uptake of 23 $\mu\text{mol m}^{-2} \text{d}^{-1}$ during periods without emissions. These soil uptake rates are in the upper range of the ones reported in these elevated CO₂ studies, but not atypical when compared to temperate grassland fluxes reported in an European [32] or global analysis [33]. Elevated CO₂ did not induce significant changes in soil moisture in our study during the time studied, and it is well possible that CH₄ fluxes remained unaltered for this reason.

The different character of CH₄ sources and sinks that contribute to the net balance of the present grassland makes it very difficult to constrain the true annual CH₄ balance of this ecosystem, for several reasons. First, sink rates due to methanotrophic activity are generally smaller than emissions rates from methanogenesis [34]. Second, while sinks are largely controlled by diffusion and continuous in time, emissions tend to be episodic because they are often mediated by ebullition, which is—on a short time scale—a discontinuous process [35]. In

Table 1. Oxidation depth (activity-weighted depth of labelling, mean \pm s.e.) in soil cores from ambient and elevated CO₂ plots, incubated under low and high CH₄ concentrations. Effects of elevated CO₂ were not statistically significant.

Date	CH ₄ concentration (ppm)	CO ₂ treatment	Oxidation depth (cm)
6 July 2011	10	ambient CO ₂	3.88 \pm 0.07
	10	elevated CO ₂	4.00 \pm 0.06
25 Oct 2011	10000	ambient CO ₂	3.81 \pm 0.08
	10000	elevated CO ₂	4.24 \pm 0.42
	10	ambient CO ₂	3.40 \pm 0.19
	10	elevated CO ₂	3.45 \pm 0.17

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the grassland investigated, the water table was relatively close to the soil surface, and it is well conceivable that the emission bursts occurred from CH₄ bubbles originating from the saturated zone. A substantial fraction of these bubbles likely travelled relatively quickly to the soil surface via preferential diffusion paths, so that this flux was not buffered. Third, the static chambers trapped localized emissions, resulting in an apparent uptake kinetic due to the re-distribution of CH₄ in the surrounding soil and possibly also an associated increase in oxidation due to the elevated CH₄ concentrations. This phenomenon is artificial and would not occur without the chamber. Finally, it is well possible that chamber handling and soil disturbance from human weight triggered the release of bubbles that would otherwise have occurred later (although the static chambers were placed carefully on the pre-installed base rings, and the weight of the person handling the chambers was distributed by a walking grid). Temporary soil compression could also have pushed high-methane air out of parts of the soil pore network where it would have stayed longer otherwise. Indeed, an indication of disturbance-triggered “burst” CH₄ release could be that the step-increase in concentrations associated with bubble emission often occurred before or just after the first headspace sampling, but rarely after the second sampling. Generally, handling-induced CH₄ release appears especially critical, since pressure variation can flush near-surface pore volumes (CH₄ fluxes: [36]; CO₂ fluxes: [37]), disturbing diffusion gradients that take long to re-equilibrate. Overall, we thus conclude that it probably is not possible to accurately assess the true CH₄ balance using static chambers in such a system, at least for periods in which net CH₄ emissions occur. One strategy may be to analyze different processes or different parts of the season independently, using different techniques (e.g. assess continuous fluxes with standard techniques and separately count the occurrence of “burst”-type events).

CH₄ fluxes exhibited marked seasonal dynamics, with emissions peaking in summer and early fall. While water table depth, soil moisture, and heavy precipitation are likely drivers of these CH₄ emissions due to their effect on oxygen supply, other factors also may have been at play. High plant activity during peak season could have supplied heterotrophic soil organisms with organic substrate, which would have lowered oxygen partial pressures when consumed—soil CH₄ oxidation, however, is generally rather limited by CH₄ concentrations unless O₂ is nearly depleted, so that seasonal dynamics are unlikely to have been affected by this mechanism. Some organic compounds can also inhibit CH₄ oxidation directly [18, 19]. Methanogenesis also is strongly temperature-dependent, and it may be that—depending on the zone in which methanogenesis occurred—sufficiently high temperatures were only reached in late summer. Finally, large numbers of Scarabidae larvae are active at the site studied, and incubations of soil cores taken from the site have previously shown that these larvae can release large amounts of CH₄ [38], a phenomenon that has not received much attention to date for temperate ecosystems.

The nature of methanotrophs capable of growing at atmospheric or sub-atmospheric CH₄ concentrations remains enigmatic, despite many years of research. Early studies have suggested that methanotrophs predominantly consuming CH₄ at low or high concentrations differ in nature [39], but it has also been argued that these organisms may be less distinct than previously thought [40]. Indeed, methanotrophs capable to adapt physiologically to environments differing in CH₄ supply have been found [5], and some possess of isoenzymes differing in kinetic properties [41]. Methanotrophs are alternately exposed to low and high CH₄ concentrations in the studied grassland, depending on whether the atmospheric or soil-internal sources dominate. Our labelling experiments suggest that the methanotrophs actively consuming CH₄ under these contrasting conditions occupy the same spatial niche. Typically, high CH₄ concentrations would be supplied from the bottom of the soil column, but our experiments showed that assimilation was nevertheless possible throughout the soil profile, so that this

likely did not bias our results. The most abundant CH₄ oxidizer at our site is a *Methylocystis* strain closely related to a cultured type (LR1) capable of displaying high-affinity kinetics when starved [42]. In this light, it appears well possible that the radiolabel assimilation we observed not only occurred at the same spatial location but that it also was driven by the same type of organisms.

The autoradiographic technique we have developed has not been applied to many sites so far. The patterns we observed, however, were similar to the ones found in the Rothamsted “Park Grass” experiment [43] and in two drought studies [9]. Labelled CH₄ assimilation concentrated in the periphery of soil features such as aggregates, probably reflecting the ease of diffusive transport to these sites. In October, when soils were wetter, CH₄ assimilating zones were more concentrated towards the soil surface, and in a smaller part of the pore network (probably macro-pores).

In conclusion, no effects of elevated CO₂ on net CH₄ fluxes and the spatial micro-distribution of methanotrophic bacteria were found in the present study. Net CH₄ fluxes were the result of CH₄ oxidation and production, with the latter dominating. There are also indications that emissions are mediated by the activity of ground-dwelling arthropods [38] and possibly fungi [44], but the mechanisms involved remain unclear. The range of sources and sinks involved, together with their different dynamic and ecological characteristics, indicate the challenges in estimating a system-level CH₄ balance and highlight the need to develop a framework in which these fluxes can be constrained; this might include analyzing periods with uptake and emissions separately, constraining these parts of the balance separately.

Supporting Information

S1 Dataset. Methane flux data presented in this article. A detailed description of the data is contained in the file.
(ZIP)

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Author Contributions

Conceived and designed the experiments: PAN CIK. Performed the experiments: SK CG. Analyzed the data: SK CG PAN. Wrote the paper: SK CG CIK PAN.

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